

Supplementary Information

Early preterm infant microbiome impacts adult learning

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Figure S1.

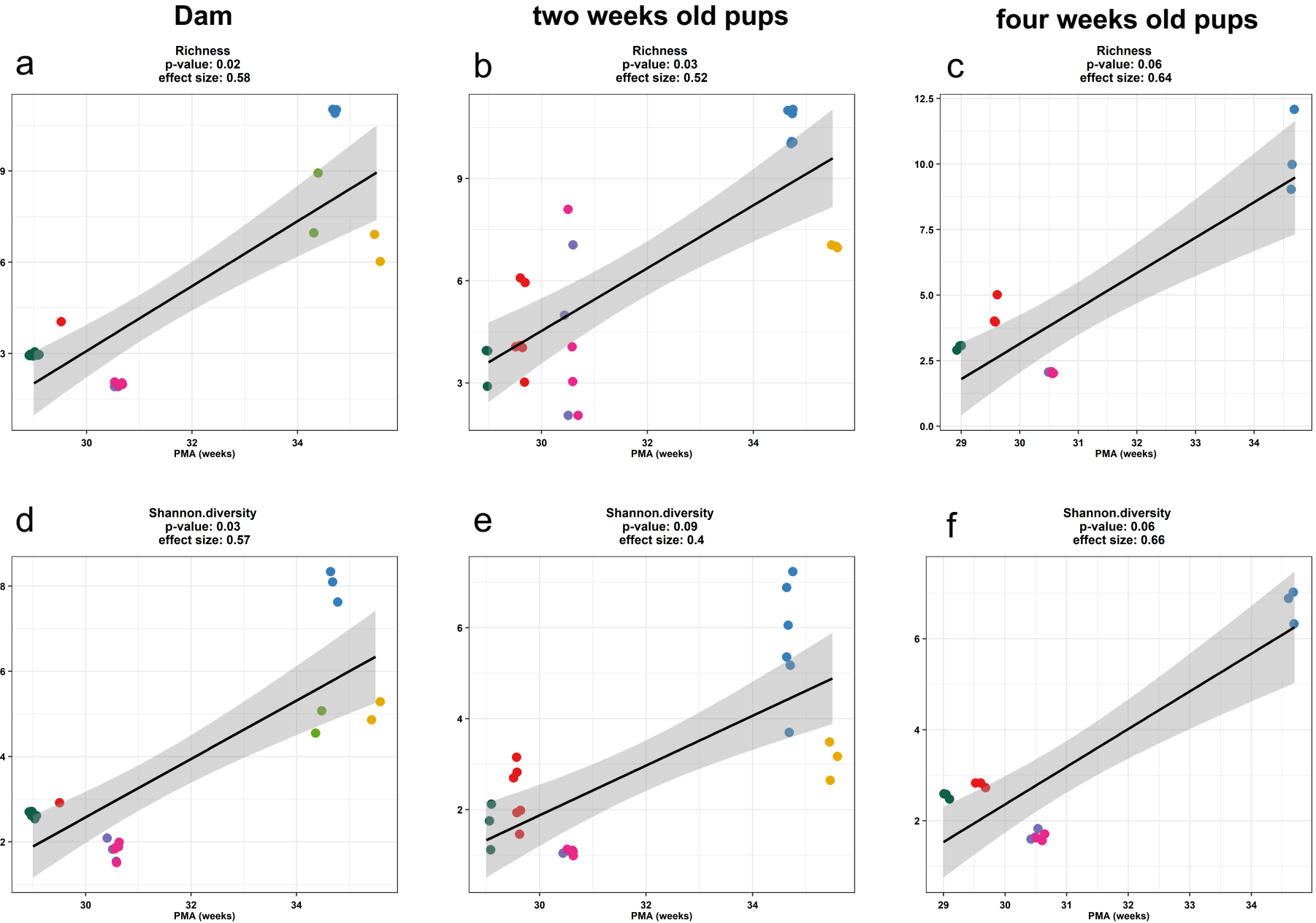


Fig. S1. Increased PMA was positively correlated with fecal microbial alpha-diversity. Pearson correlation between PMA and richness in dam (a) ($p=0.02$), two weeks old pups (b) ($p=0.03$), and four weeks old pups (c) ($p=0.06$). Pearson correlation between PMA and Shannon diversity in dam (d) ($p=0.03$), 2 weeks old pups (e) ($p=0.09$), and 4 weeks old pups (f) ($p=0.06$). Shadow area indicates a 0.95 confidence interval.

Figure S2.

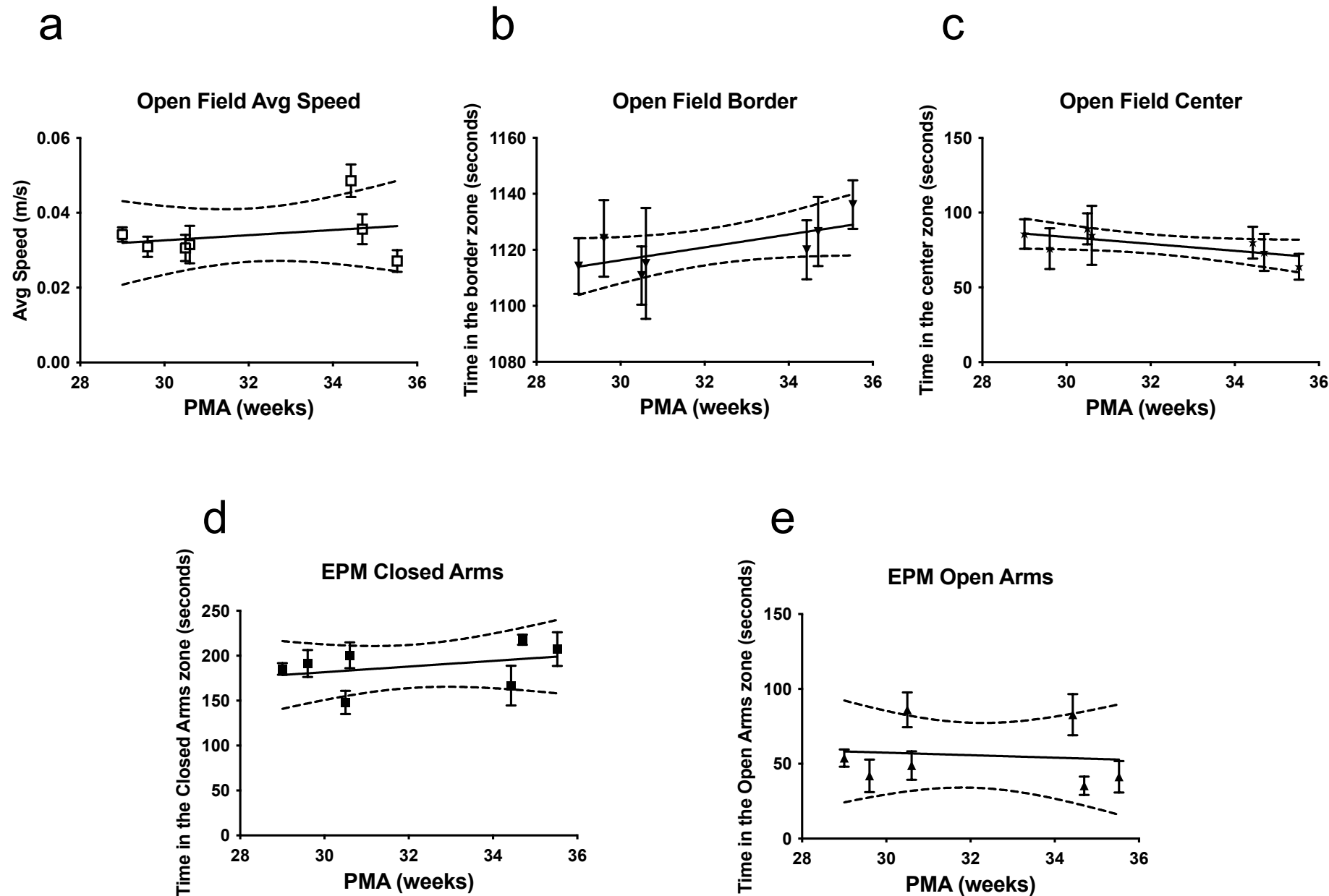
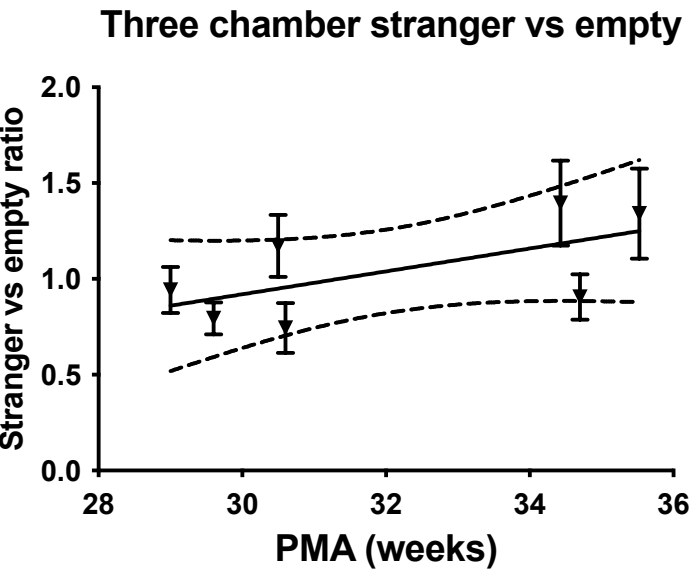


Fig. S2. Locomotor and anxiety-like activities were not affected by PMA. Average speed animals travelled (m/s), time spent in the border region of the open field (seconds), time spent in the center quadrant of the open field (seconds), time spent in the open arms of the EPM (seconds), or time spent in the closed arms of the EPM (seconds) was not related to the PMA (a-e), respectively by Pearson's correlation coefficient analysis. Animal numbers used in each transfaunation groups were: gr_1=29, gr_2=8, gr_3=14, gr_4=6, gr_5=9, gr_6=11, and gr_7=7.

Figure S3.

a



b

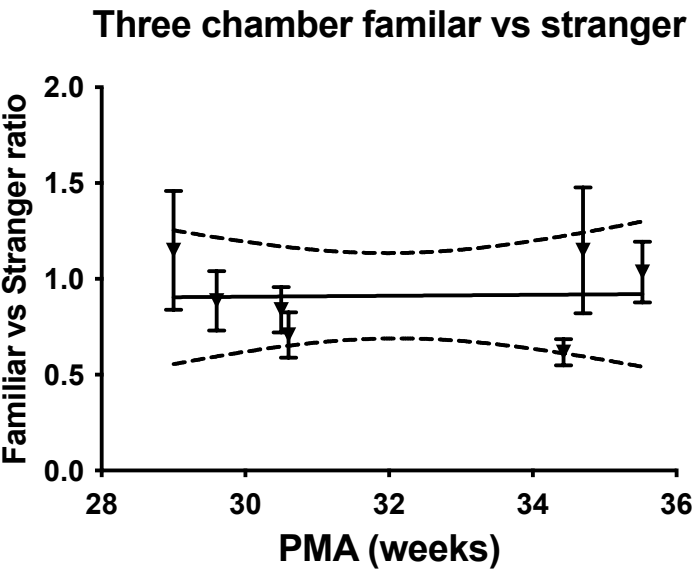
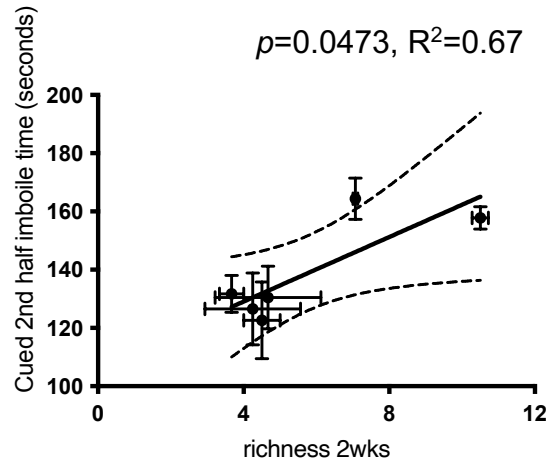


Fig. S3. Sociability and social novelty were not affected by PMA. In

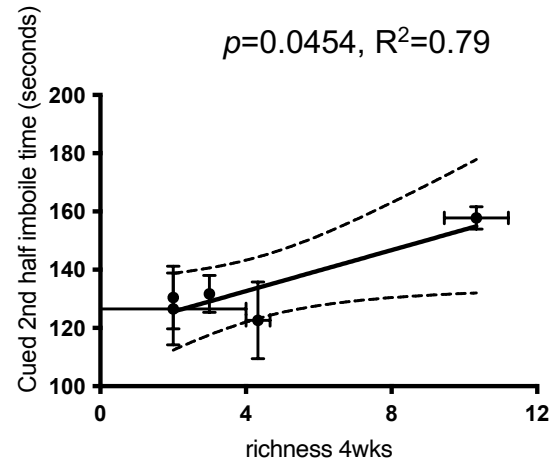
the sociability test, the ratio of the time a mouse's head was in the cup zone in which a strange mouse is placed to that in an empty cup zone was not associated with changes of PMA (a) by Pearson's correlation coefficient analysis. In the test for social novelty, the ratio of the time a mouse's head was in the cup zone in which a familiar mouse was placed to that in the cup zone of a stranger mouse was placed was not associated with changes of PMA (b) by Pearson's correlation coefficient analysis. Animal numbers used in each transfaunation groups were: gr_1=9, gr_2=8, gr_3=14, gr_4=6, gr_5=9, gr_6=10, and gr_7=7.

Figure S4.

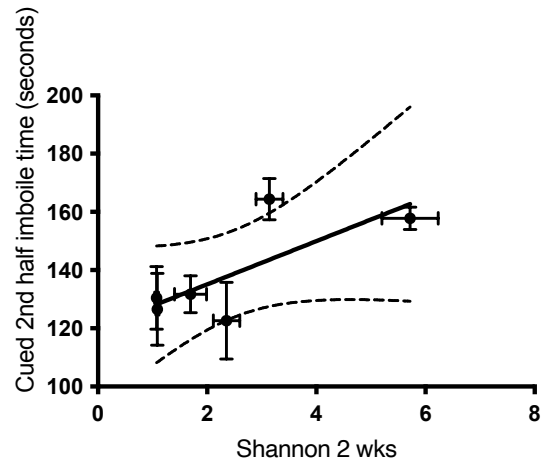
a



b



c



d

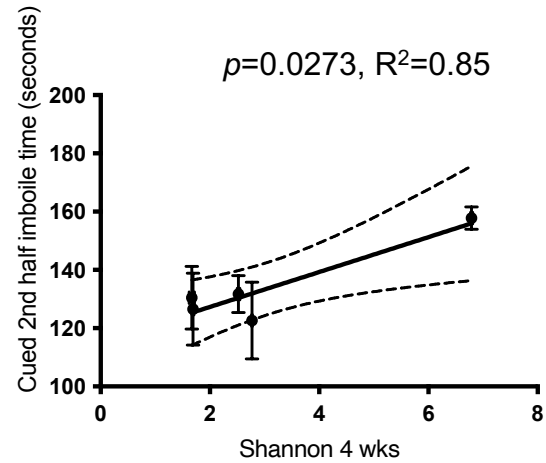


Fig. S4. Changes in richness and Shannon-diversity promoted cued fear retention in fear conditioning test. The richness of two (a) and four weeks (b) fecal samples showed a positive correlation with cued fear retention (immobile time in the second half of the cued fear conditioning test). Shannon-diversity of two weeks old fecal samples (c) was not associated with cued fear retention. Shannon-diversity of four weeks old samples (d) was correlated with cued fear retention. Pearson's correlation coefficient was considered significantly different at $p < 0.05$.

Table S1. Fecal and serum samples subjected to metabolomic analysis. PMA (week) and number (n) of fecal and serum samples were listed.

	PMA (week)	Fecal samples (n)	Serum samples (n)
Early	29.6	5	6
	30.6	5	6
Late	34.7	5	5
	35.5	5	5

Table S2. List of fecal metabolites. Fecal samples were processed according to Metabolon's platform.

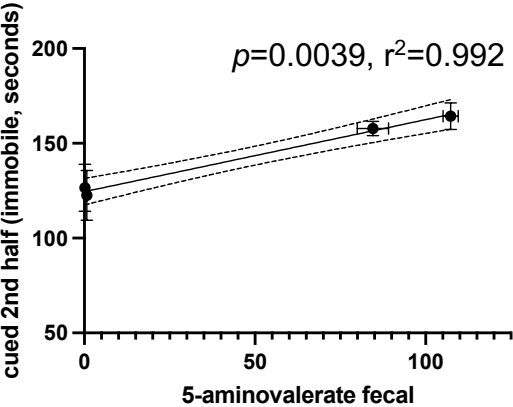
Table S3. List of serum metabolites. Serum samples were processed according to Metabolon's platform.

Table S4. Summary of Pearson correlation between PMA and metabolites. Pearson's correlation was considered significantly different at $p < 0.05$. Benjamini–Hochberg procedure was used to correct for multiple comparisons.

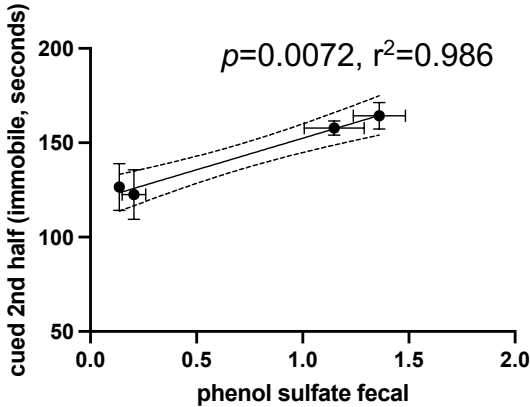
Fecal vs PMA		Fecal	Serum
5-aminovalerate	P (two-tailed)	0.0012	0.0729
	R squared	0.9435	0.8595
phenol sulfate	P (two-tailed)	0.0012	0.1212
	R squared	0.9444	0.7723
2,3-dihydroxy-2-methylbutyrate	P (two-tailed)	0.0035	0.1108
	R squared	0.9514	0.7908
ferulate	P (two-tailed)	0.0019	0.0947
	R squared	0.9299	0.8195
ursodeoxycholate	P (two-tailed)	0.0015	0.0982
	R squared	0.9385	0.8133
Serum vs PMA		Serum	Fecal
nicotinate ribonucleoside	P (two-tailed)	0.0389	0.1044
	R squared	0.9237	0.5229
beta-muricholate	P (two-tailed)	0.0457	0.0982
	R squared	0.9106	0.8133
Serum/Fecal vs PMA		Serum	Fecal
glutarate (C5-DC)	P (two-tailed)	0.0290	0.0012
	R squared	0.9428	0.9434
cholate	P (two-tailed)	0.0361	0.0160
	R squared	0.9290	0.8008
phenylacetyl glycine	P (two-tailed)	0.0460	0.0008
	R squared	0.9089	0.9554
ferulic acid 4 sulfate	P (two-tailed)	0.0187	0.0264
	R squared	0.9629	0.7471
2-oxindole-3-acetate	P (two-tailed)	0.0363	0.0037
	R squared	0.9288	0.9021

Figure 5S.

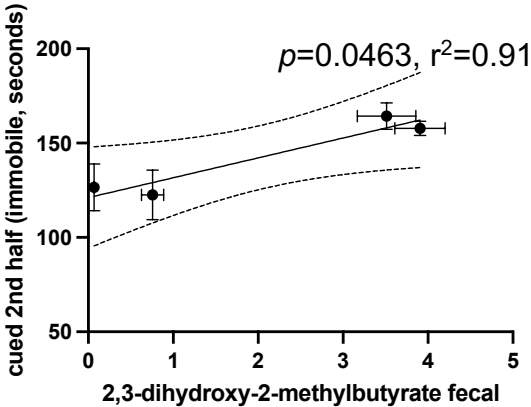
a



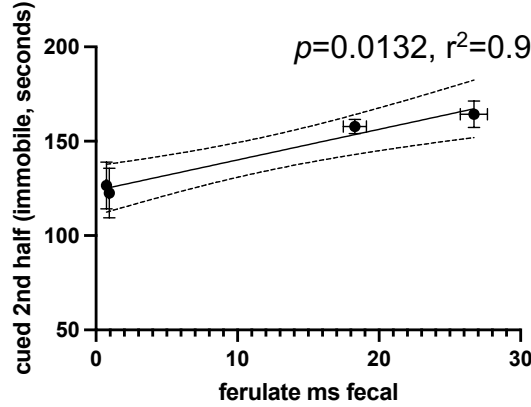
b



c



d



e

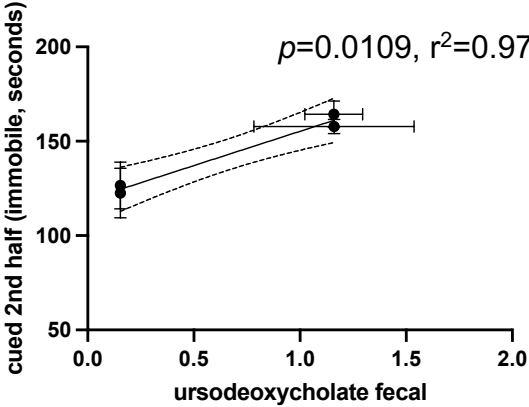
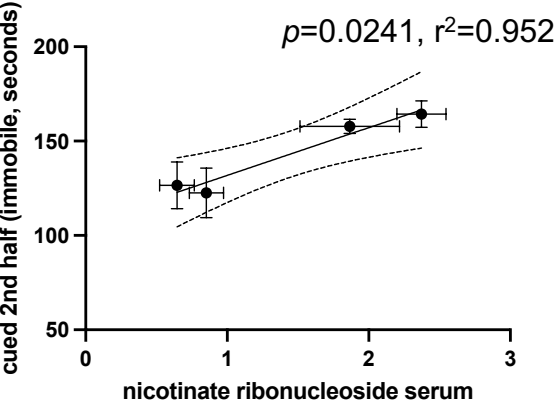


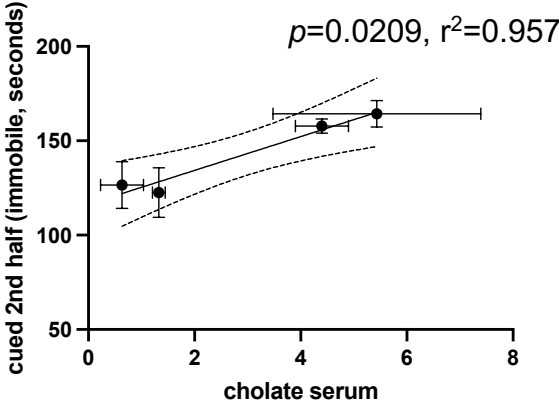
Fig. S5. Fecal metabolic features contributed to the improved fear retention. Fecal 5-aminovalerate (a), phenol sulfate (b), 2,3-dihydroxy-2-methylbutyrate (c), ferulate (d), and ursodeoxycholate (e) were positively correlated with fear retention. ScaledIMP \pm S.E.M of metabolites was used to plot against the time in the second half of the cued fear conditioning test. All reached $p < 0.05$ by Pearson's correlation analysis.

Figure 6S.

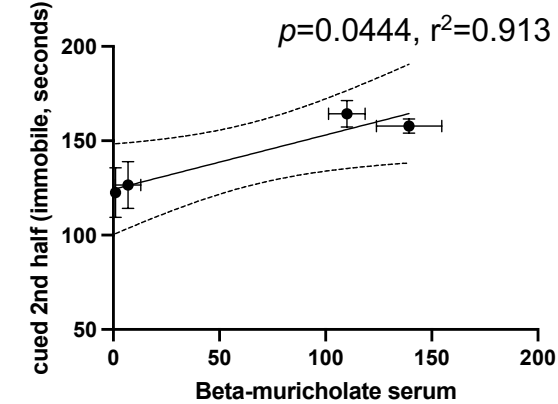
a



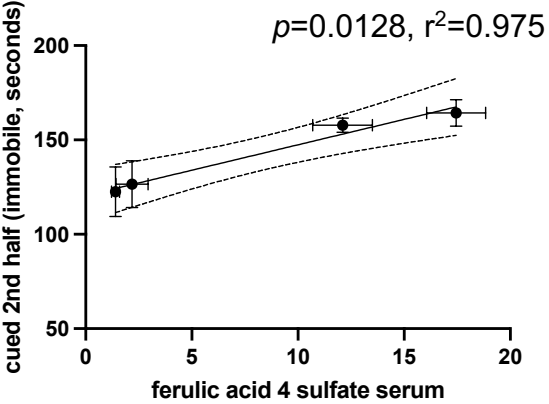
b



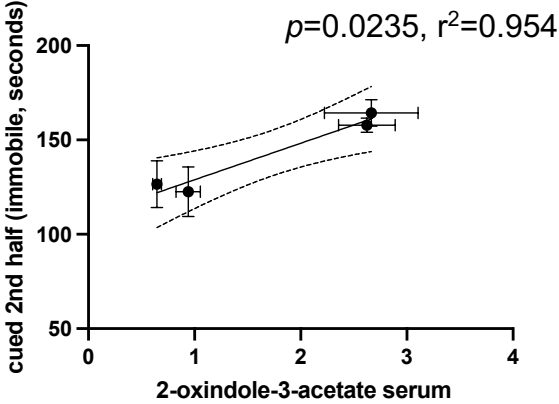
c



d



e



f

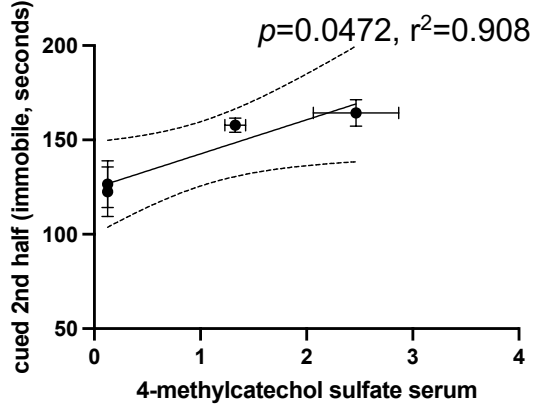


Fig. S6. Serum metabolic features contributed to the improved fear retention. Improved fear retention was positively significantly correlated with serum nicotinate ribonucleoside (a), cholate (b), beta-muricholate (c), ferulic acid 4 sulfate (d), 2-oxindole-3-acetate (e), and 4-methylcatechol sulfate (f). All reached $p < 0.05$ by Pearson's correlation analysis.

Figure 7S.

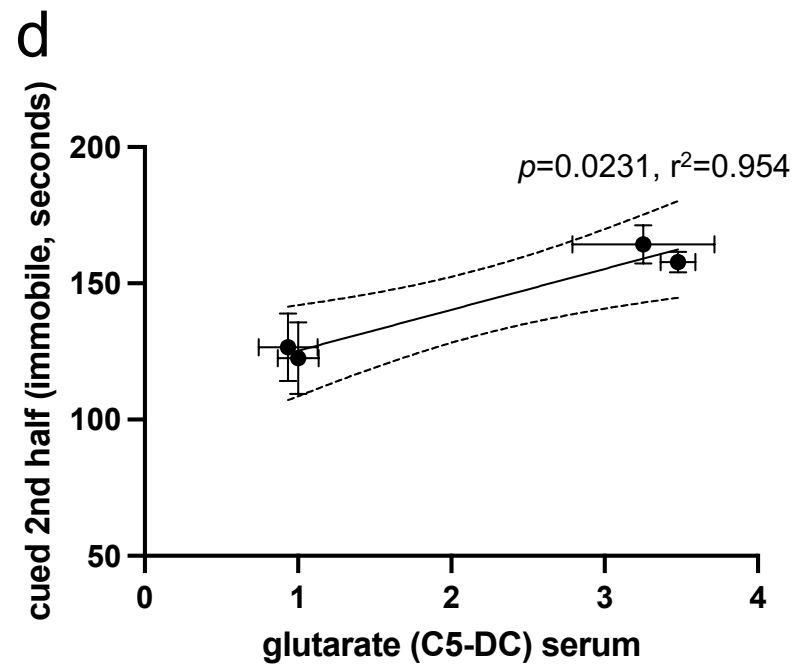
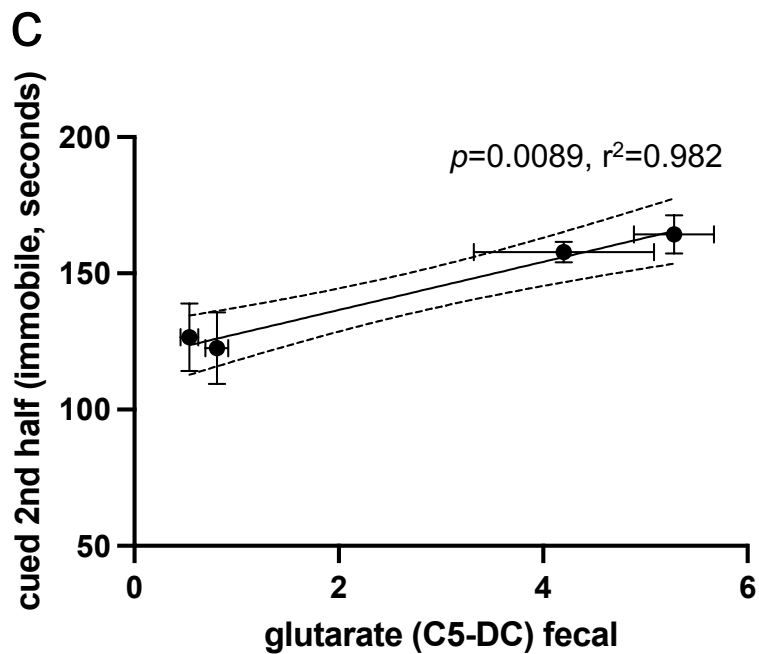
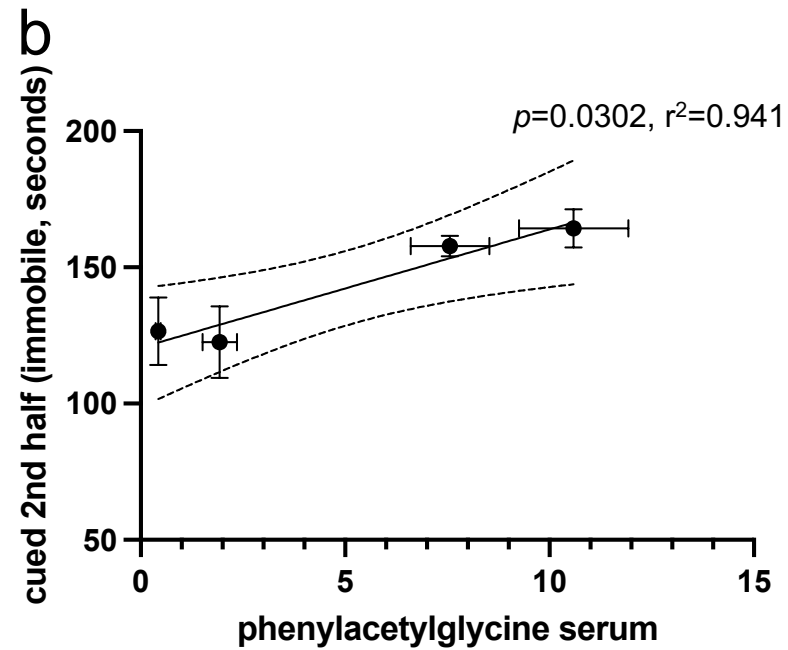
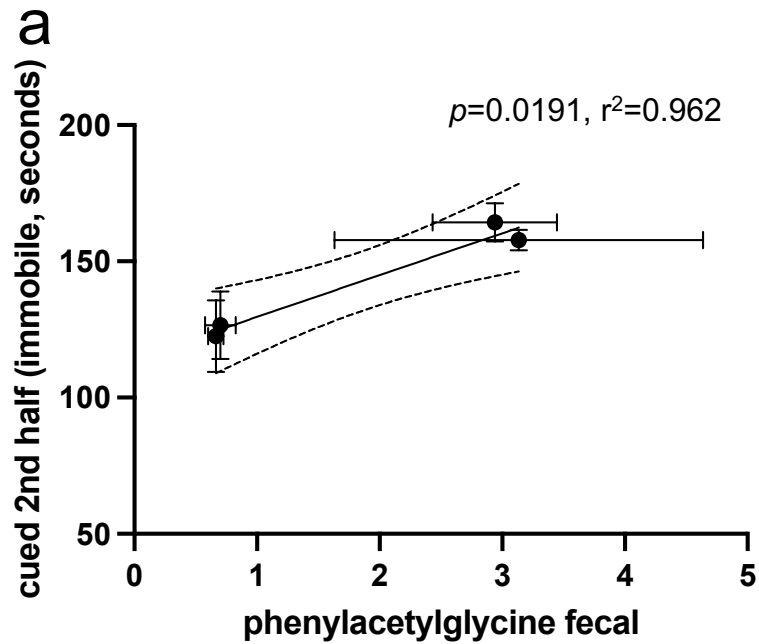


Fig. S7. Fecal and serum phenylacetylglycine (a and b) and glutarate (C5-DC) (c and d) levels were positively correlated with fear retention. All reached $p < 0.05$ by Pearson's correlation analysis.

Table S5. Number of fecal samples sequenced in different transfaunation groups and time points. Number of fecal samples in each transfaunation group from dams (dam), two weeks old pups (2wpup), and four weeks old pups (4wpup) was presented.

Group	dam	2wpup	4wpup
gr_1	8	3	3
gr_2	1	6	3
gr_3	3	3	3
gr_4	5	4	3
gr_5	2	0	0
gr_6	3	6	3
gr_7	2	3	0

Figure 8S.

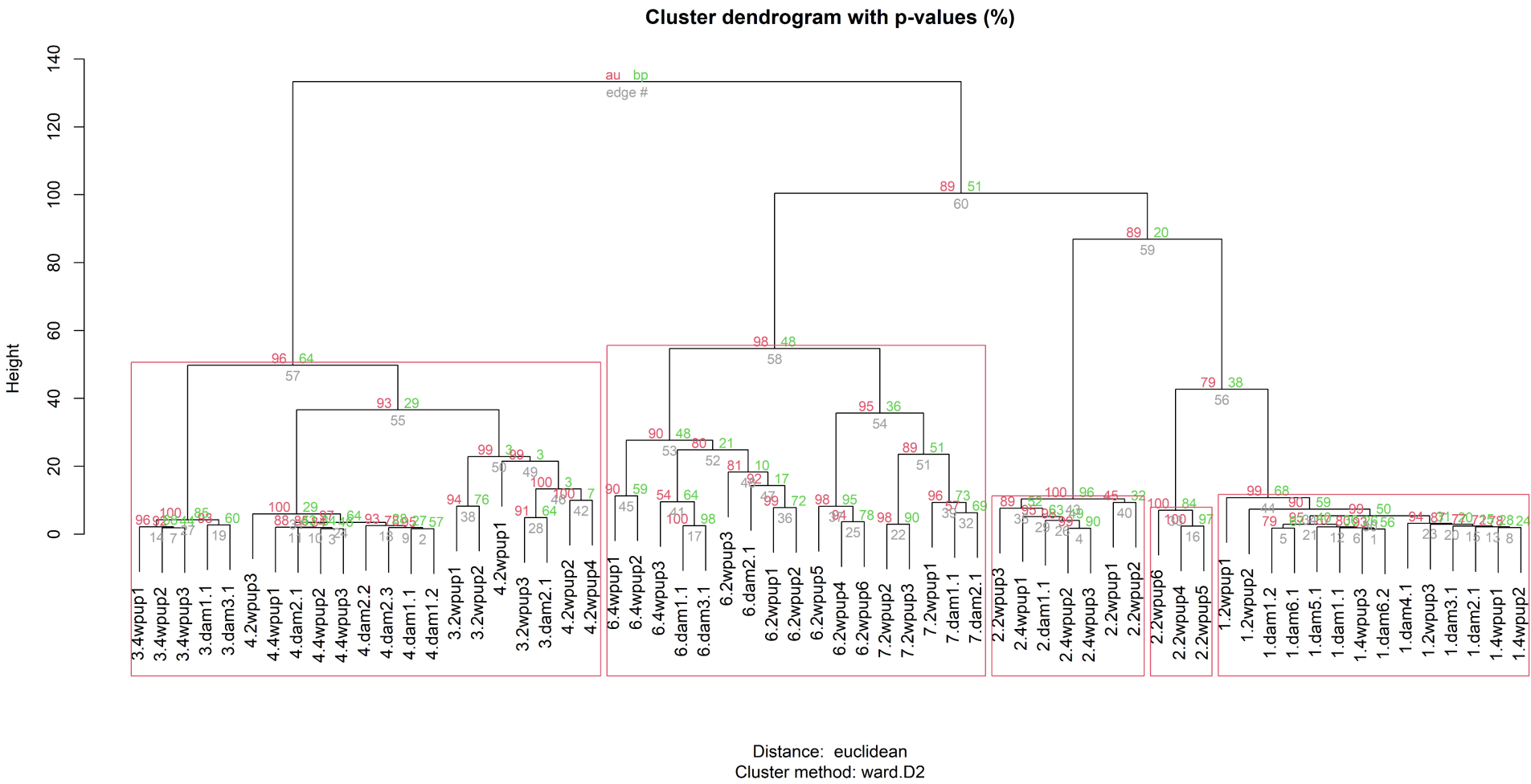


Fig. S8. Cluster analysis of fecal samples. Hierarchical clustering analysis of the Aitchson distance matrix (Euclidean distance matrix of center-log ratio transformed data) generated from fecal microbial genera abundances with Ward's minimum variance linkage method. The statistically significant clusters ($\geq 95\%$) are indicated by red rectangles, with p-values calculated from multiscale bootstrap resampling conducted by R package pvclust version 2.2-0. The approximately unbiased (AU) p-values for each edge are indicated in red text, with the ordinary bootstrap probability (BP) values in green text and cluster labels in grey text. Each sample is labeled with the convention "transfaunation group #"."sample type""sample number". Sample types are 2-week-old pup (2wpup), 4-week-old pup (4wpup) and dam. Dam sample numbers have the mouse number indicated first followed by the sample number (e.g., "7.dam2.1" is the first fecal sample from the second dam in transfaunation group 7), whereas each pup sample came from a unique pup. Pup sample #s 1-3 and all dam samples were 16S rRNA gene sequenced at the University of Chicago Duchossois Family Institute Microbiome Metagenomics Facility (Chicago, IL, USA), whereas pup sample #s 4-6 were 16S rRNA gene sequenced at the Argonne National Laboratory Environmental Sample Preparation and Sequencing Facility (Lemont, IL, USA).